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Microbial transformations of steroids-XI. Progesterone transformation by *Streptomyces roseochromogenes*–purification and characterisation of the 16α-hydroxylase system

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Abstract

Streptomyces roseochromogenes, NCIB 10984, contains a cytochrome P450 which, in conjunction with two indigenous electron transfer proteins, roseoredoxin and roseoredoxin reductase, hydroxylates exogenous progesterone firstly to 16α-hydroxyprogesterone and thereafter in a second phase bioconversion to 2β,16α-dihydroxyprogesterone. The progesterone 16α-hydroxylase P450 and the two electron transfer proteins have been purified to homogeneity. A reconstituted incubation containing these three purified proteins and NADH, the natural electron donor, produced identical hydroxy-progesterone metabolites as in intact cells.

Peroxy and hydroperoxy compounds act in a shortened form of the cycle known as the 'peroxide shunt' by replacing the natural pathway requirement for the electron donor NADH, the electron transfer proteins and molecular O_2, the terminal electron acceptor. In an $NaIO_4$ supported incubation, the initial rate of progesterone hydroxylation was marginally higher (1.62 mmol progesterone/mmol P-450/h) than in the reconstituted natural incubation (1.18 mmol progesterone/mmol P-450/h) but the product yield was significantly lower, 0.45 mol hydroxyprogesterone produced/mol P-450 compared to 6.0 mol hydroxyprogesterone produced/mol P-450. These yield data show that in the reconstituted natural pathway, progesterone 16α-hydroxylase P450 supports multiple rounds of hydroxylation in contrast to a likely single oxygenation by a minority of P450s in the peroxide shunt pathway.

Keywords: *Streptomyes roseochromogenes*; Progesterone 16α-hydroxylation; Cytochrome P450; Cytochrome P450 electron transfer proteins

1. Introduction

The genus *Streptomyces* is a rich source of cytochrome P450 monoxygenase enzymes that are involved in a wide variety of biosynthetic and xenobiotic transformation reactions. In *Streptomyces antibioticus*, this enzyme is responsible for C-8 epoxidation of the lactone ring of the antibiotic oleandomycin [1]. The DNA sequence of the *S. antibioticus* P450 is related to the eryF gene of *S. erythraea* (*Saccharopolyspora erythraea*) which codes for a soluble cytochrome P450 (CYP107) that stereospecifically 6-hydroxylates 6-deoxyerythronolide B to erythronolide B during erythromycin A biosynthesis [2]. In *S. carbophilus* $P450_{sca}$ hydroxylates compactin to pravastatin, a tissue-selective inhibitor of 3-hydroxy-3-methylglutamyl-coenzyme A reductase [3]. The sulphonyl urea herbicide chlorimuron ethyl is metabolised by two inducible *S. griseolus* P450s, $P450_{SU1}$ (CYP105A1) and $P450_{SU2}$ (CYP105B1) [4]. *S. griseus* contains a cytochrome P450 that is inducible by the isoflavenoid genistein present in soyaflour [5]. Extracts of this organism, prepared from soya flour-induced cells and supplemented with spinach ferridoxin and ferridoxin-NADPH reductase are capable of aromatic benzylic and alicyclic hydroxylation [6]. The *ChoP* gene of *Streptomyces* sp.

Abbreviations: PAGE; polyacrylamide gel electrophoresis; P450; cytochrome P450.

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SA-COO encodes a cholesterol oxidase cytochrome P450 that has a high degree of homology with human and *Pseudomonas* P450s [7].

A plethora of *Streptomyces* species have been widely reported as excellent steroid hydroxylators. C2 and C4 hydroxylation of the phenolic steroid oestradiol is known to be P450 catalysed [6]. Skeletal sites transformed in non-phenolic steroids include ξ1, 2β, 6β, 7β, 9α 11α, 11β, 15α and 16α [for examples [8–15]] but unlike in the oestradiol bioconversion the nature of these other steroid hydroxylases is unknown. However by analogy with steroid hydroxylation in the bacterial species *Bacillus cereus* [16] and *B. megaterium* [17] a reasonable assumption is that these hydroxylases are site-selective cytochrome P450 monooxygenases.

In this paper we identify 16α-monohydroxy and 2β,16α-dihydroxyprogesterone as the transformation metabolites produced by *S. roseochromogenes* during a 72 h incubation with exogenous progesterone. We also report the purification to homogeneity of the cytochrome P450 responsible for in vitro progesterone 16α hydroxylation and the two endogenous electron transfer proteins, roseoredoxin and roseoredoxin reductase.

2. Methods

2.1. Materials

Streptomyces roseochromogenes strain 10984 was purchased from the National Collection of Industrial and Marine Bacteria (NCIB) Ltd., Aberdeen, Scotland.

Media and general chemicals were purchased from the sources previously described [18]. Epoxy activated Sepharose 6B was purchased from Pharmacia Biotech Ltd., St. Albans, Herts., UK; MIMETIC Blue 1 A6XL affinity resin was obtained from Affinity Chromatography Ltd., Freeport, Ballasala, Isle of Man, UK and Cibacron Blue FG3A and NAD-Sepharose affinity gel from Sigma Chemical Co., Poole, Dorset, UK.

2.2. Media, culture maintenance and cultivation of *S. roseochromogenes* strain 10,984

S. roseochromogenes was grown at 25°C on yeast extract-malt extract-glucose (YMG) agar slopes and plates. YMG agar contained yeast extract (4 g), malt extract (10 g), glucose (4 g) and agar (15 g)/l of deionised H_2O and pH 7.2. The organism was stored at 4°C and sub-cultured every three months. The agar was omitted from liquid YMG.

2.3. Steroid transformation by *S. roseochromogenes* strain 10,984

Steroid transformations were performed essentially as previously described for filamentous fungi [18]. For small-scale analytical experiments, cells were grown at 25°C for 48 h with continuous shaking (100 rpm in an orbital incubator) in 50 ml YMG in 500 ml conical flasks containing coiled wires to aid aeration and dispersal of biomass. After an initial 24 h of growth, progesterone (0.25 ml of a 20 mg/ml stock solution in ethanol) was added to each culture flask. After a further 24 h, cells were harvested by centrifugation in an 8 × 50 ml angle rotor spun for 30 min at 8000 rpm and 4°C in a Sorvall 5B centrifuge. The supernatant was decanted and extracted with two equal volumes of chloroform. The organic layers were collected, combined and evaporated to dryness.

To obtain sufficient pure metabolites for structure determination, progesterone transformation incubations were increased fourfold.

2.4. Determination of progesterone metabolite yields produced by *S. roseochromogenes* strain 10,984

Yields of progesterone metabolites produced by *S. roseochromogenes* were determined by TLC and HPLC. In the TLC method exactly 5 mg of progesterone was added to each incubation and dried transformation products were dissolved in exactly 100 μl HPLC-grade methanol. Equal A_{242} absorbance units of sample contained in *ca* 5 μl were spotted onto fluorescent high-performance Kieselgel 60 F_{254} TLC plates, which were developed in an ethyl acetate/ether/toluene (4:3:3 by volume) solvent system. Steroids were viewed under UV light and plates were photographed. Individual spots were scraped from the TLC plate and eluted in HPLC-grade methanol. Dried steroids were re-dissolved in exactly 1 ml of methanol and the UV absorbance at 242 nm was measured. The absolute amount of steroid present was determined by reference to a calibration curve.

In the HPLC method, progesterone metabolites were separated in 60% aqueous methanol on an analytical reverse-phase Whatman Partisil PXS 5/25 ODS column. Column effluent was passed through an Pye Unicam PU 4020 UV detector set at 254 nm. Metabolite concentrations were calculated from the areas of the individual peaks eluting from the column measured on a Hewlett Packard Integrator.

2.5. Purification and structure determination of progesterone metabolites produced by *S. roseochromogenes* strain 10,984

Metabolites for structure determination were puri-

fied as described above from scaled-up incubations. Analyses were by ^{1}H NMR spectroscopy on a Bruker WH400 MHz spectrometer as previously described [18]. 16α-Hydroxyprogesterone was identified by the 'fingerprint' method [18], which involved superimposing spectra of authentic monohydroxy steroid standards on the spectrum of putative 16α hydroxyprogesterone. This assignment was confirmed by measuring chemical shifts of key substituent protons of 16α-hydroxyprogesterone and comparing these values with those in authentic monohydroxyprogesterones [19]. A full range of dihydroxyprogesterone spectra was not available to permit identification of 2β,16α-dihydroxyprogesterone by fingerprinting. The structure of this metabolite was assigned by the chemical shift calculation method described above for 16α-hydroxyprogesterone and by a full 2-D ^{1}H COSY spectrum analysis (homonuclear correlation spectroscopy) [18].

2.6. Purification of progesterone 16α-hydroxylase cytochrome P450 from S. roseochromogenes strain 10,984

S. roseochromogenes was grown in 200 ml batches as described above. Cell pellets were resuspended in buffer A (0.1 M Na phosphate, 10 mM EDTA, pH 7.2) containing 10% glycerol. Washed cells were harvested and the pellets obtained were pressed between several sheets of Whatman 3MM chromatography paper to remove excess moisture and the resulting cake was stored at −70°C.

Cells were disrupted by adding an equal mass of acid-washed sand to partially thawed biomass and the mixture was suspended in 1.5 ml ice-cold buffer A + 10% glycerol/g cell biomass. The mixture was blended at full speed in an ice-cold MSE propeller style homogeniser in six bursts of 30 sec duration. The slurry was centrifuged at 15,000 rpm for 30 min at 4°C to remove particulate matter. The supernatant (S15) was decanted and stored at −70°C.

All purification procedures were performed at 0–4°C. S15 fraction (10 ml) was loaded onto a Whatman DE52 DEAE-cellulose anion exchange column (100 ml) previously washed and equilibrated in buffer B (0.1 M Na phosphate, 5 mM EDTA, 0.25 mM DTT and 10% glycerol). The column was washed with 2 bed volumes of buffer B before proteins were eluted stepwise in buffer B containing 100, 200 and 300 mM NaCl. Each fraction was assayed for progesterone 16α-hydroxylase activity as described below.

The 200 mM DE-52 fraction, containing progesterone 16α-hydroxylase activity, was dialysed overnight against two changes of buffer B. This fraction was then loaded onto an 11α-hydroxyprogesterone coupled Sepharose 6B affinity column (11 ml) which was washed and equilibrated in buffer B. Unbound protein was washed from the column in two bed volumes of buffer B. Bound proteins were eluted in three separate fractions of buffer B containing 100, 150 and 200 mM NaCl. The 150 mM NaCl fraction contained the hydroxylase activity. This fraction was dialysed as described above.

The dialysed progesterone affinity fraction was loaded onto a MIMETIC Blue 1 A6XL column (15 ml). This column was washed with buffer B followed by buffer B containing 100 mM NaCl to remove all non-specifically bound protein. Progesterone 16α-hydroxylase activity was eluted with 120 mM NaCl in buffer B.

2.7. Cytochrome P450-dependent progesterone hydroxylation

The progesterone 16α-hydroxylase activity of purification fractions was determined using the $NaIO_4$ method as previously described [23–25]. Progesterone (4 mM) and $NaIO_4$ (1.5 mM) were added to 0.5 ml of fraction contained in metal capped Bijou bottles. Mixtures were made up to 1 ml with buffer B. Bottles were fixed to a turn-table that was vertically rotated at 40 rpm for 2 h at 25°C. Steroid metabolites were extracted from the incubations by shaking the mixtures with chloroform (1 ml) for 1 min. The chloroform layer was removed and evaporated at 60°C. The residue was dissolved in methanol (10 μl) and spotted onto TLC plates. These were run and processed as described above.

In the natural reconstituted system in which the $NaIO_4$ was replaced by NADH, purified progesterone 16α-hydroxylase cytochrome P450, roseoredoxin and roseoredoxin reductase (e.g. Table 6), hydroxylation was absolutely specific for NADH. NADPH did not substitute at any concentration tested. The NADH optimum for hydroxylation in these assays was 2 mM.

Steroid metabolites synthesised in the above incubations were identified only by TLC because the tiny quantities of compound produced precluded full chemical or NMR structural analysis. The TLC spots obtained co-chromatographed with spots of the authentic 16α-monohydroxy- and 2β,16α-dihydroxyprogesterone produced by intact *S. roseochromogenes*.

2.8. Production of reduced cytochrome P450 carbon monoxide difference spectra and determination of cytochrome P450 concentration

The cytochrome P450 concentrations of purification fractions were measured from difference spectra in a split beam Pye Unicam PU8800 spectrophotometer. Equal volumes of a P450-containing fraction were pipetted into two matched glass cuvettes. A few grains

of solid sodium dithionite were added to both fractions to reduce the P450. The cuvettes were gently inverted until the dithionite had dissolved, after which they were simultaneously scanned between 390 to 520 nm to obtain a zero baseline. Carbon monoxide was bubbled for 1.5 min into the test cuvette at a rate of 1 bubble/s. The CO difference spectrum was then obtained by re-scanning between 390 to 520 nm.

To determine the concentration of cytochrome P450 in a particular fraction, the absorbance of that fraction was measured from the difference spectrum at 450 and 490 nm. The values obtained were applied to the formula:

$$\text{P450 concentration} = \frac{\text{A450}_{\text{nm}} - \text{A490}_{\text{nm}}}{\varepsilon_{\text{o}}}$$

where ε_{o} is the millimolar absorbance coefficient of $P450_{450-490}$ and is 91 cm^{-1}/mM^{-1}.

2.9. Purification of electron transfer proteins from S. roseochromogenes strain 10,984

The roseoredoxin reductase (ferredoxin reductase) was purified from the flow-through wash of the DE-52 DEAE-cellulose column obtained in the first stage of P450 purification. This fraction was directly loaded onto a Whatman DE-32 DEAE-cellulose column and eluted with a linear 0–300 mM NaCl gradient made up in buffer B and 10% glycerol. Fractions (5 ml) were collected and assayed for cytochrome c reductase activity at 550 nm and for NADH oxidation at 340 nm. Also, the spectrum of each fraction was recorded from 260 to 460 nm and compared to that of a standard of authentic FAD.

Fractions which reduced cytochrome c, oxidised NADH and contained FAD, were pooled and dialysed for 8 h against buffer B before loading on to a Cibacron Blue FG3A column and eluted with a linear 0–200 mM NaCl gradient in buffer B. Fractions (2.0 ml) were collected and assayed as described above. Active fractions that contained roseoredoxin reductase were pooled, dialysed against buffer B + 20% glycerol (buffer C) and stored at −70°C.

The roseoredoxin reductase DE-32 cellulose column fractions obtained above were used to purify roseoredoxin (ferredoxin), the second electron transfer protein in the *S. roseochromogenes* progesterone 16α-hydroxylase cytochrome P450 pathway. Roseoredoxin activity was assayed by measuring the rate of cytochrome c reduction in the presence of NADH and purified roseoredoxin reductase (Cibacron Blue fraction). The stimulation of the basal rate of electron transfer, from roseoredoxin reductase directly to cytochrome c, was used to measure roseoredoxin activity. Active fractions were pooled and dialysed as described above and then applied to an NAD-Sepharose affinity column. Proteins were eluted with a linear 0–100 mM KCl gradient in buffer B. KCl buffers were required due to loss of electron transfer activity in NaCl. Fractions were assayed for cytochrome c reductase activity as described above and the absorbance of these fractions was measured at 280 and 414 nm. Active fractions containing the highest A414/A280 ratios were pooled, dialysed against buffer C and stored at −70°C.

2.10. Measurement of S. roseochromogenes roseoredoxin and roseoredoxin reductase activity

Roseoredoxin activity was measured in a final volume of 1 ml containing 100 mM Na phosphate, 5.2 mM $MgCl_2$ buffer pH 7.2, 0.05 mM cytochrome c, 0.05 units roseoredoxin reductase and 0.2 mM NADH. (1 unit of roseoredoxin reductase is the amount of protein required to reduce 0.5 μmol of cytochrome c per min in the presence of excess roseoredoxin). The rate of cytochrome c reduction was measured at 550 nm against a control incubation without NADH. Cytochrome c reduction was measured to completion of the reaction at 25°C. The absolute amount reduced was calculated by measuring the absorbance difference between the test and control incubations at 550 nm. An absorbance coefficient of 29.9 mM/cm was used to calculate reduced cytochrome c and 8.9 mM/cm for oxidised cytochrome c.

Roseoredoxin reductase activity was measured as described above except that roseoredoxin was omitted from the incubations.

2.11. SDS-polyacrylamide gel electrophoresis

SDS-PAGE (15% by wt) was performed according to the method of Laemmli [20] except that the running buffer contained glycerol (10% by volume). Gels were run at 125 V for 3 h, stained with Coomassie Brilliant Blue and if appropriate, over-stained with silver [21].

2.12. Protein determinations

Protein concentrations were determined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hemel Hempstead, Herts., UK).

2.13. Coupling of 11α-hydroxyprogesterone to Sepharose 6B

Freeze dried epoxy-activated Sepharose 6B (1 g) was swollen by suspending in distilled water (100 ml) and gently stirring for 5 min. The gel was harvested by filtration onto a scintered glass funnel under vacuum. The gel was then washed for 1 h with distilled water (1200 ml) before coupling to 11α-hydroxyprogesterone.

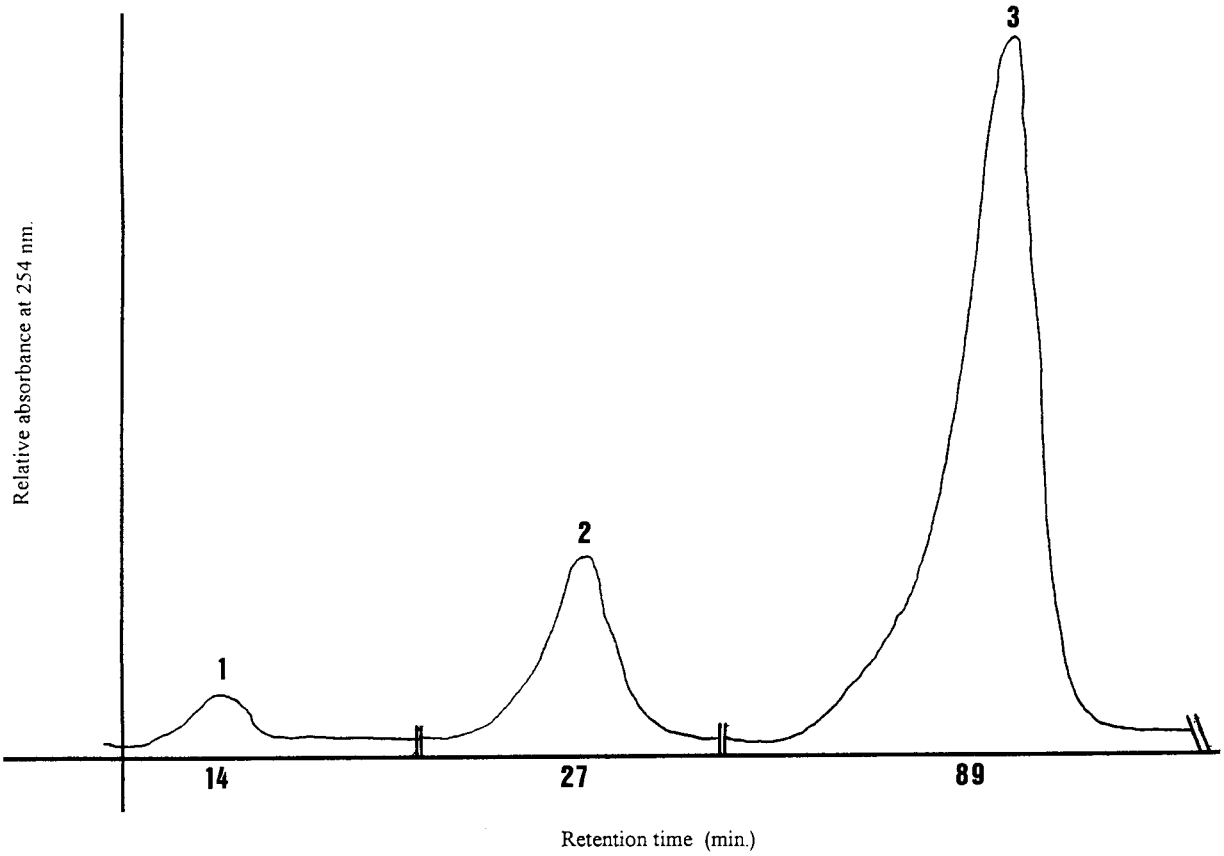

Fig. 1. HPLC of 25 h progesterone transformation incubation by *S. roseochromogenes*. From left to right, peak 1,2β,16α-dihydroxyprogesterone (retention time 14 min); Peak 2, 16α-mono-hydroxyprogesterone (retention time 27 min); Peak 3, progesterone (retention time 89 min).

The coupling was performed by adding Sepharose 6B to 1.8 mg of 11α-hydroxyprogesterone dissolved in coupling buffer containing 100 ml dimethylformamide + 100 ml 0.1 M Na phosphate buffer pH10. The suspension was incubated for 16 h at 32°C. After washing the coupled gel with copious phosphate buffer, unreacted epoxy groups were blocked by incubation in 1.0 M ethanolamine at 40°C for 10 h. The gel was again washed with phosphate buffer and the slurry poured into a glass column. The column was washed in turn with three bed volumes of 0.1 M acetate, 0.1 M NaCl buffer pH 4.0, 0.1 M borate, 0.5 M NaCl buffer pH 8.0 and finally with buffer B.

Table 1
Progesterone and its 2β and 16α-hydroxylated derivatives and HPLC peak 1 progesterone metabolite[a]

Compound	4-H	17α-H	18-H	19-H	21-H	C*H*OH
Progesterone	5.73	2.54	0.67	1.20	2.13	
2β-Hydroxy-progesterone	5.82 (0.09)	2.54 (0.00)	0.67 (0.00)	1.18 (−0.02)	2.13 (0.00)	4.20 (2α-H) (1.85)
16α-Hydroxy-progesterone	5.75 (0.02)	2.54 (0.00)	0.68 (0.01)	1.19 (−0.01)	2.18 (0.05)	4.86 (16β-H) (2.67)
2β,16α-Dihydroxy-progesterone[c]	5.84 (0.11)	2.54[b] (0.00)	0.68 (0.01)	1.17 (−0.03)	2.18 (0.05)	4.20 (2α-H) (1.85) 4.86 (16β-H) (2.67)
HPLC peak 1	5.83 (0.10)	2.54[b] (0.00)	0.67 (0.00)	1.18 (−0.02)	2.18 (0.05)	4.19 (2α-H) (1.84) 4.88 (16β-H) (2.69)

[a] δ, Relative to Me_4Si. Increments relative to progesterone in parentheses.
[b] δ calculated from the summed individual group increments, relative to progesterone.
[c] doublet.

Table 2
Production of 16α-monohydroxy- and 2β,16α-dihydroxyprogesterone by *Streptomyces roseochromogenes*

Time (h)	Total metabolite (% initial progesterone)	2β,16α-DHP (mM)	2β,16α-DHP (% initial progesterone)	2β,16α-DHP (% total metabolites)	16α-HP (mM)	16α-HP (% initial progesterone)	16α-HP (% total metabolites)
1–5	0	0	0	0	0	0	0
6	0.28	0	0	0	0.011	0.275	100
7	0.75	0	0	0	0.03	0.75	100
8	1.53	0.01	0.25	16.3	0.061	1.28	83.66
9	1.95	0.01	0.25	12.8	0.078	1.7	87.18
10	2.90	0.02	0.5	17.2	0.116	2.4	82.76
11	3.75	0.04	1.0	26.7	0.15	2.75	73.33
12	6.6	0.054	1.35	20.5	0.264	5.25	79.55
13	11.05	0.062	1.55	14.0	0.442	9.5	85.97
14	11.8	0.072	1.80	15.3	0.472	10	84.75
15	14.75	0.1	2.50	17.0	0.59	12.25	83.05
16	15.9	0.11	2.75	17.3	0.636	13.15	82.7
17	17	0.13	3.25	19.1	0.68	13.75	80.88
18	19.25	0.17	4.25	22.1	0.77	15	77.92
19	19.85	0.174	4.35	21.9	0.794	15.5	78.09
20	21.50	0.22	5.50	25.6	0.86	16	74.42
21	23.45	0.258	6.45	27.5	0.938	17	72.49
22	24.08	0.263	6.58	27.3	0.963	17.5	72.67
23	24.95	0.278	6.95	27.9	0.998	18	72.14
24	25.28	0.281	7.03	27.8	1.011	18.25	72.19

3. Results

3.1. Identification of progesterone metabolites produced by S. rosechromogenes strain 10,984

Two metabolites were purified from a 25 h progesterone transformation incubation and are shown in the HPLC trace in Fig. 1. These metabolites were identified by ^{1}H NMR spectroscopy as 2β,16α-dihydroxyprogesterone (Fig. 1 peak 1, retention time 14 min) and 16α-monohydroxyprogesterone (Fig. 1 peak 2, retention time 27 min). The NMR identifying features of 16α-hydroxyprogesterone have been described previously [22].

3.2. NMR data for 2β,16α-dihydroxyprogesterone

The ^{1}H NMR spectrum of 2β,16α-dihydroxyprogesterone has not been previously published. Therefore, we identified this compound by matching the measured values for the chemical shifts of key identifying signals of peak 1 metabolite to theoretical values calculated for 2β,16α-dihydroxyprogesterone (Table 1). The calculations involved summing the published values of the key identifying protons in 2β- and 16α-monohydroxyprogesterone [19]. 2D COSY analysis confirmed the structure. The features of structural significance of 2β,16α-dihydroxyprogesterone are summarised below.

Peak 1 retained the fundamental signals of the progesterone skeleton, i.e. 4-H (s[1], δ 5.83), 18-H_3 (s, δ 0.67), 19-H_3 (s, δ 1.18), 21-H_3 (s, δ 2.18) and displayed the features of dihydroxylation with two clearly separated mid-field multiplets between δ 4.9 and δ 4.2 attributable to methine protons of secondary hydroxylated carbon atoms. The shape and spectral position of these peaks was consistent with 2β,16α-dihydroxylation. This transformation was confirmed by the following spectral data. The characteristic triplet of 17α-H in progesterone was coincident with a predicted doublet (δ 2.54), consequent on splitting by the 16β-H. 16α-Hydroxylation was confirmed by the strong correlation of the 17-H in the COSY with the distinctive mid-field multiplet (2.69 ppm) at δ 4.88 extant at lower field than any other CHOH proton of a hydroxyprogesterone as a result of the proximity of the 16β-H to the 20-oxo group. Correlation of COSY cross peaks of the mid-field multiplet CHOH signal at δ 4.19 with lα-H at δ 2.4, which was significantly shifted to low field (0.71 ppm) relative to progesterone and to 1β-H (δ 1.56) and unusually shifted to high field (−0.48 ppm) relative to progesterone, confirmed 2β-hydroxylation.

[1] s, singlet; m, multiplet.

3.3. Time course of progesterone transformation by S. roseochromogenes strain 10,984

16α-Hydroxyprogesterone, the major metabolite of progesterone transformation, first appeared in the culture medium after 6 h of incubation (Table 2). This metabolite was actively produced throughout the entire 24 h incubation but the rate of production significantly slowed after 22 h. A second phase metabolite, 2β,16α-dihydroxyprogesterone, was first detected in the culture medium 2 h after the first appearance of 16α-hydroxyprogesterone. This compound steadily accumulated during the next 13 h of transformation but at less than half the rate of synthesis of 16α-hydroxyprogesterone. Production also virtually ceased at 21 h. 2β-Hydroxyprogesterone, the counterpart monohydroxy metabolite to 16α-hydroxyprogesterone, was not observed at any time during the 24 h transformation period. At the end of transformation, 25% of substrate progesterone had been converted into hydroxylated products of which *ca* 72% was 16α-hydroxyprogesterone and *ca* 28% was 2β,16α-dihydroxyprogesterone.

3.4. Optimisation of progesterone hydroxylation by S15 homogenates of S. roseochromogenes strain 10,984

The conditions summarised below were established for optimal periodate hydroxylation of progesterone by *S. roseochromogenes* S15 supernatants (data not shown). The most active extracts were obtained from mature cultures grown for about 35 h and from cells disrupted by blending for 3 min with an equal mass of sand in bursts of 30 s, interspersed with 30 s resting on ice. Protease inhibitors in the blending buffer had no significant effect on hydroxylation, whereas the addition of 10% glycerol stimulated hydroxylation nearly 3-fold. EDTA concentrations to 10 mM were slightly stimulatory (30%) but were strongly inhibitory at higher concentrations. At 30 mM EDTA, hydroxylation was totally inhibited. A similar effect was observed with DTT. The optimum concentration that gave 50% stimulation was 0.5 mM and 3 mM DTT inhibited hydroxylation by 50%. The pH activity profile was extremely sharp, the optimum being pH 7.2. Hydroxylation was severely inhibited either side of this value (30% respectively at pH 7.0 and 7.6). In contrast the $NaIO_4$ oxidant optimum was broad and flat from 1.5 mM to 5 mM. A true optimum for progesterone concentration could not be determined due its poor solubility in incubation buffer.

Thus, varying the concentration from 0.5 to 7.5 mM (beyond the solubility limit) had virtually no effect on metabolite production.

Using these optimal conditions, hydroxylation was linear for up to 18 minutes at 25°C and was pro-

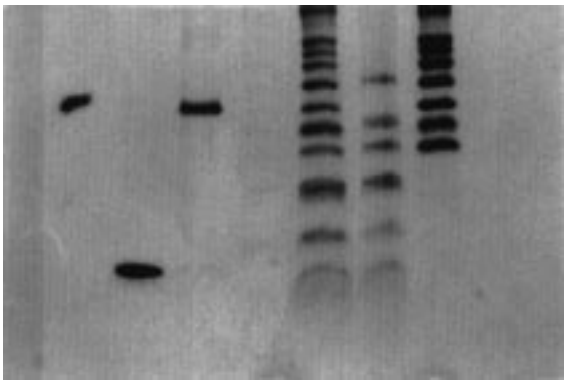

Fig. 2. SDS-polyacrylamide gel of purified *S. roseochromogenes* 16α-hydroxylase cytochrome P-450, roseoredoxin and roseoredoxin reductase. From left to right, lane 1, roseoredoxin reductase (3.1 μg M_r 65×10^3); lane 2, roseoredoxin (2.8 μg M_r 14×10^3); lane 3, progesterone 16α-hydroxylase cytochrome P450 (4 μg M_r 63×10^3); lane 4, wide range protein size ladder M_r 205×10^3–14.2×10^3; lane 5, lower range protein size ladder M_r 66×10^3–14.2×10^3; and lane 6, upper range protein size ladder M_r 205×10^3–45×10^3. Lanes 4–6 contained 35 μg total protein.

portional to S15 concentrations between 1 to 6 mg protein/ml. Hydroxylation was 50% inhibited by 1 μg/ml ketoconazole and 100% inhibited by 4 μg/ml. This ketokonazole inhibition is supportive of cytochrome P450 as the progesterone 16α-hydroxylase enzyme.

3.5. Purification of progesterone 16α-hydroxylase cytochrome P450 from S. roseochromogenes strain 10984

A three step procedure was used to purify progesterone 16α-hydroxylase cytochrome P450 to homogeneity judged by SDS-PAGE and silver staining (Fig. 2). $NaIO_4$ was used in the hydroxylation assays *in lieu* of NADH and the electron transfer proteins required in the natural P450 pathway. The details of the P450 purification are summarised in Table 3. *S. roseochromogenes* S15 extract was applied to a DE52 DEAE-cellulose column, which was eluted by a step 50–350 mM NaCl gradient. The hydroxylase activity was in the 200 mM NaCl fraction. This fraction contained less than 5% of the total protein applied to the column.

After dialysis, the DE52 fraction was applied to a progesterone affinity column and eluted with a NaCl step-gradient. The fractions obtained were assayed for haemoproteins by absorbance at 417 nm as well as for general protein (absorbance at 280 nm). Over 95% of the general protein applied to this particular column failed to bind and eluted in the flow-through and wash fractions. These fractions contained virtually no haemoprotein. The haemoprotein was found to bind moderately tightly to the progesterone affinity ligand and, together with the remaining 6.5% of protein, eluted in

Table 3
Purification of *S. roseochromogenes* progesterone 16α hydroxylase cytochrome P450

Purification stage	Total protein (mg)	P-450 (nmol)	P-450 (nmol/mg protein)	Recovery (%)	Purification factor
S15 extract	1050	21.01	0.019	100	1
DEAE ion exchange (200 mM NaCl fraction)	42.59	20.02	0.47	95.29	24.7
Dialysis	42.53	18.1	0.43	86.14	22.6
Sepharose 6B 11α-progesterone affinity column (150 mM NaCl fraction)	2.8	3.3	1.18	15.71	62.1
Dialysis	2.79	2.94	1.05	13.99	55.3
MIMETIC Blue I affinity column (120 mM NaCl fraction)	0.08	0.27	3.46	1.29	182.1
Dialysis	0.08	0.25	3.21	1.2	168.9

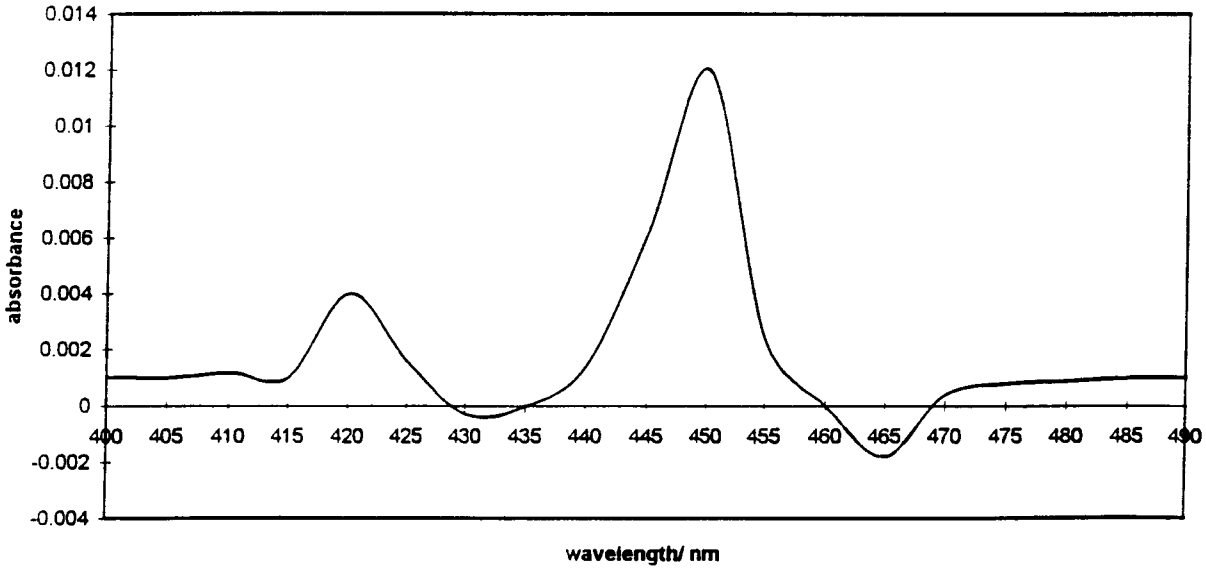

Fig. 3. Dithionite reduced, carbon monoxide difference spectrum of the 120 mM NaCl MIMETIC Blue 1 affinity column fraction. Each cuvette contained 0.79 mg P450.

150 mM NaCl. This fraction contained all the hydroxylase activity.

In the third purification step, the 150 mM NaCl progesterone affinity fraction was applied to a MIMETIC Blue 1 affinity column. This column also proved to be extremely efficient as virtually 100% of the 417 nm absorbing material initially applied, i.e. the haemoprotein, was bound but only 3% of the total protein. All bound protein and progesterone 16α-hydroxylase activity eluted in the 120 mM NaCl fraction. A single protein band was seen for this fraction on SDS-PAGE (Fig. 2). Single protein bands were also observed on haem-stained and Coomassie blue-stained 5% native acrylamide gels and on 3% isoelectric focusing gels (results not shown). An M_r value of 63×10^3 was determined for the P450 from the SDS-PAGE and a p*I* value of 6.6 from the isoelectric focusing gels.

Based on CO difference spectroscopy, a 182 fold purification and a yield of 1.3% native P450 was calculated for this simple purification procedure. A small minority of the cytochrome P450 was in inactive P420 form (Fig. 3).

3.6. Purification of the progesterone 16α-hydroxylase cytochrome P450 electron transfer proteins roseoredoxin reductase and roseoredoxin from S. roscochromogenes strain 10,984

The DE-52 DEAE-cellulose column flow-through and wash, obtained from the first stage of P450 purification, was used as starting material for the purification of the electron transfer proteins roseoredoxin reductase and roseoredoxin. A two step procedure was used to purify to homogeneity both proteins. The data for these methods are shown in Table 4 (roseoredoxin reductase) and Table 5 (roseoredoxin) respectively.

In the case of roseoredoxin reductase, a second DEAE column, containing DE32 cellulose, followed by dye affinity chromatography on Cibacron Blue FG3A resulted in a 258 fold purification of this particular protein. Roseoredoxin co-eluted with the roseoredoxin reductase in the DE32 300 mM NaCl fraction, but was separated from the latter protein by NAD-Sepharose affinity chromatography to give a final 163 fold purification. Both proteins gave single bands in SDS-PAGE (Fig. 2). M_r values of 14×10^3 and 65×10^3 were determined from the SDS-PAGE gels for roseoredoxin and roseoredoxin reductase respectively (Fig. 2).

3.7. Comparison of progesterone metabolism catalysed by a reconstituted natural cytochrome P450 dependent hydroxylation pathway and an $NaIO_4$ dependent peroxide shunt pathway

The roseoredoxin, roseoredoxin reductase and NADH requirement in the natural progesterone 16α-hydroxylase cytochrome P450 hydroxylation pathway is replaceable by an organic or inorganic peroxy or hydroperoxy compound (XOOH) such as $NaIO_4$. When these two pathways were compared for progesterone transformation catalysed by highly purified *S. roseochromogenes* progesterone 16α hydroxylase cytochrome P450, it was found that the initial rate of hydroxylation was nearly 40% greater in the $NaIO_4$ peroxide shunt pathway (1.62 mmol progesterone converted/mmol P-450/h) than in the natural reconstituted pathway (1.18 mmol progesterone converted/mmol P-

Table 4
Purification of *S. roseochromogenes* roseoredoxin reductase

Purification stage	Total protein (mg)	Total roseoredoxin reductase (nmol)	Yield roseoredoxin reductase (nmol/mg protein)	Recovery roseoredoxin reductase	Purification factor
S15 extract	1050	32	0.03	100	1
DEAE 52 column flow-through	800	30.5	0.04	95.31	1.3
DE 32 0-300 mM NaCl fraction	6.84	19.96	2.92	62.38	97.3
Dialysis	6.84	18.44	2.70	57.63	90
Cibacron Blue FG3A 0-200 mM NaCl fraction	1.88	14.55	7.74	45.47	258
Dialysis	1.88	13.03	6.93	40.72	231

Table 5
Purification of *S. roseochromogenes* roseoredoxin

Purification stage	Total protein (mg)	Total roseoredoxin (nmol)	Yield roseoredoxin (nmol/mg total protein)	Recovery roseoredoxin (%)	Purification factor
S15 extract	1050	51	0.05	100	1
DEAE 52 column flow-through	800	39.10	0.05	76.67	1
DE 32 0-300 mM NaCl fraction	6.84	18.79	2.75	36.80	55
Dialysis	6.84	17.77	2.6	34.80	52
SepharoseNAD affinity 0-100 mM KCl fraction	1.95	9.81	5.03	19.20	101
A414/A280 fraction pooled and Dialysis	1.10	8.95	8.14	17.55	163

Table 6
Comparison of progesterone metabolism catalysed by the reconstituted natural cytochrome P450 dependent hydroxylation pathway and the $NaIO_4$ dependent pathway

Pathway	Initial rate of progesterone transformation (mmol progesterone/mmol P-450/h)	Turnover (mol progesterone/mol P-450)
Reconstituted	1.18	6.00
$NaIO_4$	1.62	0.45

450/h). By contrast, the peroxide shunt pathway supported 13 fold fewer hydroxylation events per molecule of P450 (0.45 mol progesterone converted/mol P-450) than the reconstituted pathway (6.0 mol progesterone converted/mol P-450) (Table 6).

4. Discussion

S. roseochromogenes was first identified as catalysing steroid 2β- and 16α-hydroxylation 40 yr ago during the search for microorganisms capable of efficient and mild stereospecific access of the 16 site of the steroid nucleus [e.g. [23–24]]. Microbial access of this site was eventually employed for the synthesis of a new generation of highly potent, synthetic, anti-inflammatory pharmaceuticals exemplified by triamcinalone (9α-fluoro-11β,16α,17α,21-tetrahydroxy-pregna-1,4-diene-3,20-dione) developed to replace natural corticosteroids found to suffer mineralocorticoid contraindications.

S. roseochromogenes 10,984 possesses strong progesterone 2β- and 16α-hydroxylation activities. The time course of progesterone transformation (Table 2) shows 16α-hydroxylation to be the primary event and 2β-hydroxylation to be a second phase reaction using 16α-monohydroxyprogesterone as substrate. Thus, 16α-monohydroxyprogesterone was first detected in the culture medium after 6 h of incubation and 2β,16α-dihydroxyprogesterone appeared 2 h thereafter at 8 h. That this is the true route of progesterone bioconversion was shown when *S. roseochromogenes* transformed exogenous 16α-hydroxyprogesterone to 2β,16α-dihydroxyprogesterone. Moreover, free 2β-monohydroxyprogesterone was never detected in our system even in transformation incubations containing 20 mg of progesterone and a metabolite detection threshold of under 0.1 mg of steroid.

There is now a wealth of compelling evidence identifying cytochrome P450 as responsible for steroid hydroxylation reactions in both bacteria and filamentous fungi e.g. in bacteria — 6β in *Bacillus thermoglucosidasius* [25] and 15β in *Bacillus megaterium* [17]: in filamentous fungi — 7α in *Phycomyces blakesleeanus* [26]; 11α in *Aspergillus fumigatus* [27], *Aspergillus ochraceus* [28], *Nectria haematococca* [29] and *Rhizopus nigricans* [30]; 11β in *Cochliobolus lunatus* [31]; and 15α in *Penicillium raistrickii* [32]. Despite the long history and extensive literature on microbial steroid hydroxylation no such role has so far been indisputably assigned to P450 for this reaction in *Streptomyces* species. Therefore, this paper is the first report to identify unequivocally cytochrome P450 as a steroid hydroxylase enzyme in the *Streptomyces* genus.

A turnover number of *ca* 0.02/min was calculated for *S. roseochromogenes* P450 catalysed progesterone hydroxylation in the reconstituted pathway and a comparable value of *ca* 0.03/min for the $NaIO_4$ dependent pathway. Both numbers are now the lowest reported for a purified P450 being an approximate order of magnitude lower than the previous bottom of the table turnover numbers of 0.6/min reported for the steroid 15β-hydroxylase cytochrome P-450 ($P\text{-}450_{meg}$) of *Bacillus megaterium* [33] and of 1.8/min and 2.1/min reported for precocene II and 7-ethoxy coumarin hydroxylation respectively catalysed by a crude fraction of *S. griseus* P450 [6]. The reason for this catalytic inefficiency is unclear, but it may be connected with the fact that progesterone is almost certainly not the physiological substrate for the *S. roseochromogenes* P450. Thus, a meaningful comparison of the true efficiency of this enzyme with its bacterial counterparts cannot be made until a natural substrate is identified. However these data show that in the natural pathway highly pure *S. roseochromogenes* P450 catalyses multiple cycles of hydroxylation even with the unnatural steroid substrate.

In contrast to steroid hydroxylation, cytochrome P450 has been identified in several *Streptomyces* pathways of secondary metabolism. Thus, this enzyme participates in oleandomycin biosynthesis in *Streptomyces antibioticus* [1], the hydroxylation of compactin to prevastatin by *Streptomyces carbophilus* [3] and in a wide variety of xenobiotic transformations in *Streptomyces griseus* [4–6].

Cytochrome P450 genes have been cloned from only two *Streptomyces* species, *S. griseolus* and *S. griseus*. In *S. griseolus*, genes for the herbicide-inducible cytochromes P450, P450SU1 and P450SU2, have been sequenced and the amino acid sequence of segments of the encoded proteins deduced. These proteins were found to possess high sequence identity to the *Pseudomonas putida* camphor hydroxylase P450 (P450 101, $P450_{cam}$) particularly in the haem binding domain

[34]. A DNA segment carrying the structural gene encoding P-450$_{soy}$ (soyC), has been cloned from *S. griseus*. It is noteworthy that this cytochrome P450 also has high sequence conservation with P450$_{cam}$ in the haem binding region [35]. It would be interesting to compare the primary sequence of the *S. roseochromogenes* progesterone 16α-hydroxylase cytochrome P450 with those of these other *Streptomyces* P450s.

Ferredoxin electron transfer proteins have also been purified from the above two *Streptomyces* species. *S. griseolus* was found to contain two 7 kDa isoforms, designated Fd-1 and Fd-2. These proteins have 52% identity and both contain single [3Fe-4S] clusters [36]. Both ferredoxins are active in reconstituted cell-free systems containing the SU1 P-450 isoform, although Fd-2 is more effective. The genes for the ferredoxins and the sulfonylurea P450 monooxygenases are virtually contiguous. Thus P-450SU1 and the downstream Fd-1 form a closed spaced pair, similarly P-450SU2 and Fd-2. In *S. griseolus* only a single ferredoxin encoded by SOY B has been putatively identified. The situation in *S. roseochromogenes* remains to be resolved.

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